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MUTANT CHOLERA HOLOTOXIN **AS AN ADJUVANT**

HOLOTOXINE **MUTANTE DU** CHOLERA **UTILISEE COMME ADJUVANT**

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Detailed Description
Claims

English Abstract
A mutant **cholera holotoxin** featuring a point mutation at amino acid 29 of the A subunit, wherein the glutamic...

...fungus or parasite. In a particular embodiment, the amino acid 29 is histidine. The mutant **cholera holotoxin** may contain at least one additional mutation in the A subunit at a position other...

...acid 29. The antigenic composition may include a second adjuvant in addition to the mutant **cholera holotoxin** .

French Abstract
L'invention concerne une **holotoxine** mutante du **cholera** comprenant un point de mutation au niveau de l'acide amine 29 de la sous...

...d'acide glutamique est substitue par un acide amine autre que l'acide

aspartique. Cette **holotoxine** mutante du **cholera** est utilisee comme adjuvant dans une composition antigenique afin d'ameliorer, chez un vertebre, la...

...un parasite. Dans un mode de realisation, l'acide amine 29 est l'histidine. Cette **holotoxine** mutante du **cholera** peut contenir au moins une mutation additionnelle dans la sous-unite A au niveau d'une position autre que l'acide amine 29. Outre l' **holotoxine** mutante du **cholera** , cette composition antigenique peut comprendre un second adjuvant.

Detailed Description

MUTANT **CHOLERA** HOLOTOXIN AS AN AD = ANT

Field of the Invention

This invention relates to the use of an immunogenic mutant **cholera** **holotoxin** having reduced toxicity compared to a wild-type **cholera** toxin and a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the **cholera** **holotoxin** as an adjuvant to enhance the inmune response in a vertebrate host to a selected...

...with an immunogen or antigen is known as an adjuvant.

The Gram-negative bacterium Vibrio **cholerae** (V. **cholerae**) is the causative agent of the gastrointestinal disease **cholera** . The diarrhea caused by V. **cholerae** is due to the secretion of **cholera** toxin (CT),

CT comprises a single A subunit (CT-A), which is responsible for the...

...ganglioside GM, on their surface. Together, the CT-A and CT-B subunits comprise a **holotoxin** . The sequence of CT has been described (Bibliography entry 1).

CT a is hexaheteromeric complex...

...adjuvant.

It would be preferable to use as an adjuvant a form of the CT **holotoxin** that has reduced toxicity so as to reduce the undesirable symptoms of diarrhea caused by wild-type CT. Thus, there is a need to identify a mutant CT **holotoxin** which is able to enhance the immune response while reducing the toxicity of the CT **holotoxin** .

Summary of the Invention

Accordingly, it is an object of this invention to utilize a mutant form of the CT **holotoxin** that has reduced toxicity compared to a wild-type CT as an adjuvant in an...

...bacterium, virus, fungus or parasite.

These objects of the invention are achieved with a mutant **cholera** **holotoxin** featuring a point mutation at amino acid 29 of the A subunit, wherein the

glutamic...

...immune response of a vertebrate host by including an effective adjuvanting amount of a mutant **cholera holotoxin** , wherein the **holotoxin** has reduced toxicity compared to a wild-type CT and the glutamic acid at amino acid position 29 of the A subunit of the **cholera holotoxin** is replaced by an amino acid other than aspartic acid, in particular a histidine.

The which encode an immunogenic mutant **cholera holotoxin** having a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the **cholera holotoxin** , and wherein such a DNA sequence is operatively linked to an arabinose inducible promoter, as...

...as to suitable host cells transformed, transduced or transfected with such plasmids. The immunogenic mutant **cholera holotoxin** is produced by transforming, transducing or transfecting a host cell with a plasmid described above in the A subunit (CTCRME29H). The cumulative data demonstrate that CT CRME29H is a **holotoxin** and is less toxic than wild-type CT, Importantly, CT-CRME29H is able to augment...

...The mutant CT-A retained its ability to assemble with CT-B to form a **holotoxin** that resembled wild-type CT in its adjuvanticity, but exhibited reduced toxicity compared to a...
...type CT and the glutamic acid at position 29 of the A subunit of the **cholera holotoxin** is replaced by an amino acid other than aspartic acid. In a particular embodiment of this invention, the amino acid 29 is histidine.

As used herein, the term "the **holotoxin** has reduced toxicity" means that the CT-CRM mutant, such as the CT-CRME29H mutant...

...variant CT-A's R7K, E29H, E1IOD and E112D were able to assemble into immunoreactive **holotoxin** as determined by a ganglioside GM, binding assay (Figure 1). However, a portion of purified R11K did not appear to be a **holotoxin** when tested with the polyclonal antibodies described in Example 2.

Each **holotoxin** variant was tested in a Y-1 adrenal tumor cell assay (19) to determine its residual toxicity compared to wild-type CT **holotoxin** . The results presented in Table 2 demonstrated that CT CRME29H and commercial CT-B (Sigma...
...cell and ADP ribosylation activity assays are due to trypsin - 12 activation of the mutant **holotoxin** in the latter assay, Thus, the lack of CT-A cleavage into Al and A2...

...coli-expressed CT-CRM.29,' Collectively, the accumulated data show

that CT-CRME29H is a **holotoxin** that binds to ganglioside GM, and is significantly less toxic than wild-type CT.

A...5). The antibody titers of mice administered the recombinant - 13 proteins plus wild-type CT **holotoxin** were elevated 20 fold. The anti-rP4 antibody titers of mice immunized with the CT...protein antibody responses is correlated (r = 0.97) with the antibody response to the mutant **cholera holotoxin** .

In this ninth experiment, mucosal immunity was also assessed, Mucosal IgA was observed only in...made to express CT-CRME29H in E.coli. The resulting yield of purified CT-CRME29H **holotoxin** was approximately 50@Lg per liter of culture medium. Initial attempts to increase CT-CRM...

...moderate increase in yield was achieved through co expression of pIIB29H, and derivatives, with Vibrio **cholerae** DsbA and E. coli RpoH. Co-expression and purification modifications increased the yield of CTCRME29H...

...CRME29Mo During cloning it was determined that plasmid pIIB29H contained a ctxA gene from Vibrio **cholerae** strain 569B, linked to a ctxB gene from V.c. strain 2125. Cross alignment of...

...genes which leads to an amino acid change within the A-2 portion, or the **holotoxin** assembly domain of the A subunit, It was not known whether the heterogeneity between these genes had a negative impact on toxin expression or **holotoxin** assembly; however, it was thought preferable from an evolutionary standpoint that both toxin subunit genes...

...plasmids containing isolated and purified DNA sequences comprising DNA sequences which encode an immunogenic mutant **cholera holotoxin** having a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the **cholera holotoxin** , and wherein such a DNA sequence is operatively linked to an arabinose inducible promoter, as...

...A variety of host cell-plasmid vector systems are used to express the immunogenic mutant **cholera holotoxin** . The vector system, which preferably includes the arabinose inducible promoter, is compatible with the host...the DNA encoding the CT-CRM is expressed by the host cell.

The immunogenic mutant **cholera holotoxin** is produced by transforming, transducing or transfecting a host cell with a plasmid described above...

...as asparagine, cysteine, glutamine, glycine, serine, threonine and tyrosine.

An effective amount of the mutant **cholera holotoxin** , wherein the **holotoxin** has reduced toxicity compared to a wild-type **cholera holotoxin** and has a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the **cholera holotoxin** , in combination with a selected antigen from a pathogenic bacterium, virus, fungus or parasite, is used to prepare an antigenic composition, wherein said **holotoxin** enhances the immune response in a vertebrate host to said antigen.

The antigenic compositions of...

...series of mutations in the A subunit which serve to reduce the toxicity of the **cholera holotoxin** . These mutations include making substitutions for the arginine at amino is acid 7, the aspartic...

...and the arginine at position 192, The nucleotide sequence encoding the A subunit of the **cholera holotoxin** is set forth in International application WO 93/13202. International application WO 98/42375 (37...

...amino acid 109 in the A subunit, which serves to reduce the toxicity of the **cholera holotoxin** . Therefore, using conventional techniques, mutations at one or more of these additional positions are generated...

...be mixed with immunologically acceptable diluents or carriers in a conventional manner.

The immunogenic mutant **cholera holotoxin** of this invention is suitable for use as an adjuvant in antigenic compositions containing a...ml; kanamycin 25 gg/ml; tetracycline 10 jAg/ml). A complete CT operon from V. **cholerae** 0395 was subcloned into the phagemid vector pSKII-, under the control of the lac promoter...

...The plasmid encoding CT-CRME29H is designated pIIB29H, The plasmid contains the polycistron of V.

cholerae genes ctxA and ctxB which encode CT, The ctxA gene in this plasmid was mutagenized...

...sequences were replaced with the signal sequence-encoding region of E. coli LT (LTIIb-B **leader**) in order to promote secretion of CT-CRM,2,,' The plasmid pIIB29H was then modified...

...of ctxA and ctxB, The two genes are genetically separated in pPX2492, unlike in V. **cholerae** , where the genes overlap. The two genes also have the LTIIb-B **leader** sequence upstream of each.

EMression of mutant ctxA alleles

Production of each variant **holotoxin** was tested in 5 ml cultures of TB medium (45) in 125 ml Erlenmeyer flasks...Cells were removed by centrifugation, and the supernatants were assayed to determine the concentrations of **holotoxin** and B pentamer as described below.

Specifically, the production of CT-CRME29H in E. coli involves the co-expression of the genes rpoH from E. coli and dsbA from V. **cholerae** . These gene products participate in the conformational maturation of both the A and B subunits of CT,

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Example 2

The GMI Binding Assay for Intact **Holotoxin**

The CT-CRMs were examined in a ganglioside GM1@dependent solid phase radioinmunoassay (42) to determine whether intact. **holotoxin** was present after purification. An enzyme-linked immunosorbent assay (ELISA) was used where ELISA plate...

...CT-CRMs with amino acid substitutions at positions 7, 29, 110, or 112 were intact **holotoxins** (Figure 1). The results implied, however, that a portion of purified CT-CRMR1IK did not appear to be a **holotoxin** .

Example 3

Y-1 Adrenal Cell Assay for Residual Toxicity of CT-CRMs

The mutant CT-CRMs were compared several times with wild-type **holotoxin** for toxicity in the mouse Y-I adrenal tumor cell assay. Y-1 adrenal cells...

...CT-CRM multiplied by 100, Table 2 depicts the residual toxicity of several purified mutant **holotoxins** tested in the Y-1 adrenal cell assay.

Table 2

The toxicity for Y-1...of BALB/c mice immunized awith recombinant P4 and P6 proteins b formulated with mutant **cholera** **holotoxins**

Serum Anti'-Recombinant P4 IgG Antibody Titersc

Adjuvant d Day 0 Day 21 Day 35...

...of BALB/c mice immunizeda with recombinant P4 and P6 proteins b formulated with mutant **cholera** **holotoxins**

Serum Anti-Native P6 IgG Antibody Titersc

Adjuvant d Day 0 Day 21 Day 35...

...antibody responses of BALB/c mice immunizedawith recombinant P4 and P6 proteinsb formulated with mutant **cholera** **holotoxins**

Anti-Recombinant P4 Antibody Titersc

NW d BAWd VWd

e

Adjuvant IgA IgG IgA IgG...similar fragment from plasmid pMGJ142 which was shown to encode a ctxB gene from V. **cholerae** strain 569B, The resulting construct,

pPX7490 encodes the CT-CRME29H ctxA and ctxB genes from strain 569B under control of the arabinose promoter, and has the LTIIb-B **leader** sequence.

Protocols for the large scale expression and purification of CT-CRME29H were developed and...

...0) at 0.255 cm/min (5 ml/min) to remove contaminants, The CT-CRME29H **holotoxin** was eluted with four column volumes of 10mM NaPO4 (pH 8,3) at 0.255...F., et al., "ADP-Ribosylation Factors: A Family of Guanine Nucleotide-Binding Proteins that Activate **Cholera** Toxin and Regulate Vesicular Transport", pages 257-280 in Handbook of Natural Toxins: Bacterial Toxins...

Claim

... a pathogenic bacterium, virus, fungus or parasite and an effective adjuvanting amount of a mutant **cholera** **holotoxin** , wherein the **holotoxin** has reduced toxicity compared to a wild-type **cholera** **holotoxin** and has a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the **cholera** holotoxin , wherein said **holotoxin** enhances the immune response in a vertebrate host to said antigen.

2 The antigenic composition...

...composition of Claim 1 which further comprises a second adjuvant in addition to the mutant **cholera** **holotoxin** .

15 The antigenic composition of Claim 1 wherein at least one additional mutation is made to the A subunit of the **cholera** **holotoxin** at a position other than amino acid 29,

16 The antigenic composition of Claim 15...

...an isolated and purified DNA sequence comprising a DNA sequence which encode an immunogenic mutant **cholera** **holotoxin** having a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the **cholera** **holotoxin** , and wherein the DNA sequence is operatively linked to an arabinose inducible promoter. - 122

25...

...transfected with the plasmid of Claim 24.

26 A method of producing an immunogenic mutant **cholera** **holotoxin** , wherein the **cholera** **holotoxin** has reduced toxicity compared to a wild-type **cholera** **holotoxin** and has a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the **cholera** **holotoxin** , which comprises transforming, transducing or transfecting a host cell with the plasmid of Claim 24...

...detoxified protein by the

host cell.

27 Use of effective adjuvanting amount of a mutant **cholera holotoxin** , wherein the **holotoxin** has reduced toxicity compared to a wild-type **cholera holotoxin** and has a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the **cholera holotoxin** , in combination with a selected antigen from a pathogenic bacterium, virus, fungus or parasite, to prepare an antigenic composition, wherein said **holotoxin** enhances the immune response in a vertebrate host to said antigen.

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